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ScienceDirect
Veterinary Immunology and Immunopathology 115 (2007) 334–345
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

Differential response of splenic monocytes and DC from cattle to microbial stimulation with *Mycobacterium bovis* BCG and *Babesia bovis* merozoites

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Received 17 August 2006; received in revised form 25 October 2006; accepted 2 November 2006

Abstract

Both bovine peripheral blood monocyte-derived dendritic cells (DC) and myeloid DC from afferent lymph have been described, but resident DC from other bovine tissues have not been fully characterized. The spleen as a secondary lymphoid organ is central to the innate and acquired immune response to various diseases particularly hemoprotozoan infections like babesiosis. Therefore, we developed methods to demonstrate the presence of myeloid DC from the spleen of cattle and have partially characterized a DC population as well as another myeloid cell population with monocyte characteristics. The phenotypic profile of each population was $CD13^+CD172a^{+/-}CD14^-CD11a^-CD11b^{+/-}CD11c^+$ and $CD172a^+CD13^{+/-}CD14^+CD11a^-CD11b^{+/-}CD11c^+$, respectively. The $CD13^+$ population was found exclusively in the spleen whereas the $CD172a^+$ population was present at the same percentage in the spleen and peripheral blood. $CD13^+$ cells developed a typical veiled appearance when in culture for 96 h. The two cell populations differed in their ability to produce nitric oxide and had a different pattern of cytokine mRNA when stimulated with *Mycobacterium bovis* BCG or *Babesia bovis* merozoites. The data demonstrate the presence of a myeloid splenic DC with attributes consistent with an immature status.

Keywords: Bovine; Splenic dendritic cell; Immature dendritic cell; Cytokines; Nitric oxide

1. Introduction

Dendritic cells (DC) are professional antigen-presenting cells (APC) that have a central role in the initiation and regulation of immune responses. DC are able to prime naïve lymphocytes and bias the immune response toward T-cell helper 1 (Th1) or T-cell helper 2 (Th2) responses (Banchereau et al., 2000). The study of distinct DC populations is crucial because it may lead to a better understanding of the initial immunological events related to protection against microbial infection.

Studies in humans and mice have identified lymphoid and myeloid DC populations (Liu, 2001; Shortman and Liu, 2002; Wu and Dakic, 2004). It has been proposed that lymphoid DC are dedicated to the adaptive immune system whereas myeloid DC act as an interface between the innate and adaptive immune systems (Gluckman et al., 2002; Della et al., 2005).

Distinct populations of myeloid DC have been demonstrated in humans and mice in different tissues, including skin (Larregina et al., 2001), bone marrow (Egner and Hart, 1995), blood (Gluckman et al., 2002),

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doi:10.1016/j.vetimm.2006.11.001

lymph nodes (Henri et al., 2001; Wu and Dakic, 2004), spleen (Henri et al., 2001; Wu and Dakic, 2004), and Peyer's patch (Kelsall and Strober, 1996; Iwasaki and Kelsall, 2000). Studies in cattle using afferent lymph veiled cells showed the existence of heterogeneous populations of myeloid DC that differ in their ability to stimulate T cells and pattern of cytokine expression (Howard et al., 1997, 2004; Stephens et al., 2003). Although distinct populations of myeloid DC share some features, such as expression of MHC class II and co-stimulatory molecules, they differ in phenotype markers and function, and these differences may determine the fate of the T cells they activate (Gluckman et al., 2002). In addition, the presence of heterogeneous myeloid DC populations suggests that distinct subsets may be specialized for certain immunological functions.

Our laboratory has developed a technique to study cell populations from the spleen of cattle. In this technique, the spleen is marsupialized affording the opportunity to aspirate and isolate cells from the organ sequentially over a prolonged period of time (Goff et al., 1991). Using this technique we carried out experiments to isolate a distinct myeloid DC population not present in peripheral blood.

2. Material and methods

2.1. Cattle

Three Holstein–Friesian steer calves were obtained at 8 weeks of age. The animals were vaccinated against pathogenic *Clostridium* species, castrated and dehorned. All animals were tested negative for *Anaplasma marginale* and *Babesia bovis* by ELISA (Torioni de et al., 1998; Goff et al., 2003b). The calves were maintained according to the American Association for Laboratory Animal Care procedures with acceptable bovine ration, water and mineral block provided *ad libitum*. At 12 weeks of age, each animal underwent a surgical procedure to marsupialize the spleen (Varma and Shatry, 1980). The marsupialized animals were used until 8 months of age as a source of spleen mononuclear cells (SMC) and peripheral blood mononuclear cells (PBMC).

2.2. Preparation of mononuclear cells from spleen and peripheral blood

SMC were obtained from marsupialized animals as previously described (Goff et al., 1996). Briefly, approximately 35 ml of spleen tissue was aseptically aspirated into 60 ml syringes containing 15 ml acid-citrate-dextrose (ACD) pH 7.3. The spleen aspirate was processed into a single cell suspension using a tissue homogenizer and approximately 25 ml layered onto 20 ml of Hypaque-Ficoll (1.086 g/l, Accupaque, Accurate Chemicals, Westbury, NY, USA). The samples were centrifuged for 30 min at 1500 × *g* at 4 °C and the SMC interface from similar gradients were collected, pooled and washed in 50 ml of Dulbecco's Modified Eagle's Medium (DMEM, pH 7.35). The cells were centrifuged for 7 min at 400 × *g* at 4 °C, washed two to three times in DMEM and residual erythrocytes, when present, were lysed by AKC lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3). After the washes, SMC were suspended in Iscove's medium (Gibco BRL, Gaithersburg, MD, USA) containing 2 μM glutamine, 10 μg/ml gentamicin, 50 μM mercaptoethanol, and 15% fetal bovine serum, counted and used for flow cytometry and positive selection.

To obtain PBMC, peripheral blood was centrifuged for 10 min at 1500 × *g* at 4 °C, and the buffy coat collected and washed in DMEM as described for SMC. PBMC were suspended in Iscove's medium, counted and used for flow cytometry procedures.

2.3. MAbs, cytokines and microbial antigens

Table 1 describes the antigen, identification, isotype and source of leukocyte differentiation-specific monoclonal antibodies (mAbs) used in this study. Recombinant bovine IFN-γ (rBo-IFN-γ) was a generous gift of Drs. Loren Babiuk and Dale Godson, Vaccine and Infectious Disease Organization, Saskatoon, Saskatchewan, Canada, and was used at 50 U/ml. Recombinant human TNF-α (rHu-TNF-α) (R&D Systems) was used at 2500 U/ml. For microbial stimulant experiments, heat-killed *Mycobacterium bovis* BCG (HK-BCG) (50 μg/ml) and viable *B. bovis* merozoites (BBO-MZ) were used. BBO-MZ were isolated as previously described (Goff et al., 1998a), and used at a ratio of 10 per 1 leukocyte.

2.4. Isolation of $CD13^+$ and $CD172a^+$ populations

The mAbs CC81 and CC149 were utilized to isolate $CD13^+$ cells and $CD172a^+$ cells from fresh SMC, respectively. The two cell populations were isolated using MACS microbeads (Miltenyi Biotec, Auburn, CA, USA) following the manufacture's instructions. Briefly, SMC were incubated with either mAb CC81 or CC149 (5 μg per 10^8 cells) for 10 min at 4 °C. Subsequently, cells were washed once in PBS without Ca^{2+} and Mg^{2+}, pH 7.0, supplemented with 0.5% BSA and 2 mM EDTA and incubated with 100 μl of goat

Table 1
Description of the antigen recognized, name, isotype and source of the monoclonal antibodies to leukocyte surface markers used in this study

Antigen	Antibody	Isotype	Source
CD14	CAM36A	IgG1	VMRD, Pullman, WA, USA
CD14	MM61A	IgG1	VMRD, Pullman, WA, USA
CD13	CC81	IgG1	Institute for Animal Health, Compton, UK[a]
CD205 (WC6)	CC98	IgG2b	Institute for Animal Health, Compton, UK[a]
CD11a	IL-A99	IgG2a	Washington State University, Pullman, WA, USA[b]
CD11b	MM10A	IgG2b	VMRD, Pullman, WA, USA
CD11b	BAQ147A	IgM	VMRD, Pullman, WA, USA
CD11c	BAQ153A	IgM	VMRD, Pullman, WA, USA
CD172a	CC149	IgG2b	Institute for Animal Health, Compton, UK[a]
CD172a	DH59B	IgG1	VMRD, Pullman, WA, USA
MHCII	TH14B	IgG2a	VMRD, Pullman, WA, USA

[a] Provided by Chris Howard.
[b] Provided by Bill Davis.

anti-mouse IgG microbeads (Miltenyi Biotec) for 15 min at 4 °C. MACS LS columns were used to select the cell populations. The cells were then suspended in Iscoves's medium, counted, and the purity of the two selected populations evaluated by FACS.

For microbial and cytokine stimulation experiments, the two cell populations were plated at 10^5 cells per well in 96-well plates or chamber slides (Lab-Tek, Nalge Nunc International, Rochester, NY, USA) and cultivated at 37 °C, 5% CO_2. After incubation with either cytokines or microbial agents, the cells were collected at different time points and either Giemsa stained for morphometric analysis or used for RT-PCR. The cell supernatants were checked for the presence of nitric oxide.

To confirm the presence of Fc receptors, normal and antibody-sensitized sheep red blood cells (RBC) were incubated with each cell population at a 10 RBC to 1 leukocyte ratio for 30 min on ice. The cells were then cytocentrifuged at 28.23 × *g* for 3 min and stained by Giemsa and examined microscopically.

2.5. Flow cytometry for phenotype analysis

Fresh SMC, PBMC, $CD13^+$ cells and $CD172a^+$ cells were labeled with a panel of surface mAbs (Table 1) using standard one-, two- or three-color flow cytometry procedures. Briefly, 1–2 × 10^7 cells were incubated with 5 μg/ml of appropriate mAbs for 15 min on ice. The cells were washed three times with PBS containing 10 mM EDTA, 0.2% sodium azide, 10% ACD, and 2% γ-globulin-free serum and incubated for 15 min on ice with appropriate isotype-specific fluorescein-, phycoerythrin-, and tri-color-conjugated goat anti-mouse Igs (Caltag, Burlingame, CA, USA). The cells were washed three times as described before and analyzed by FACScan™ flow cytometry (Becton Dickinson, Mountain View, CA, USA) with a minimum of 5000 data points per sample. All flow cytometry data were analyzed with either CellQuest software (Becton Dickinson) or FCS Express (De Novo Software, Thornhill, Ontario, Canada).

2.6. RT-PCR for cytokines and iNOS expression

$CD13^+$ or $CD172a^+$ populations were incubated at different time points with HK-BCG, BBO-MZ or medium, and then cellular RNA was isolated using 1 ml TRIzol (BRL, Bethesda, MD, USA) and 20 μg/ml glycogen (Boehringer Mannheim, Germany). RNA samples were used for RT-PCR amplification of IL-1β, IL-10, IL-12 p40, IL-12 p35, IFN-γ, TNF-α, iNOS, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as previously described (Goff et al., 1998a). RNA samples were reverse transcribed by Superscript II Reverse Transcriptase (BRL) using oligo-$(dT)_{12}$ as primer. Specific cDNA sequences were amplified by PCR using Taq DNA polymerase (BRL) and the primers described in Table 2. The PCR products were resolved on 2% NuSieve (FMC Bioproducts, Rockland, ME, USA) agarose gels containing ethidium bromide. The level of mRNA for each cytokine and iNOS from each sample was determined by densitometry image analysis, where the GAPDH measurement was used as the standard (IS-1000 Digital Imaging System and Alpha-EASE 3.21 software program, San Francisco, San Leandro, CA, USA). Progressive GAPDH cDNA dilutions were evaluated and optimized by densitometry to confirm the reliability of the method as a semi-quantitative measure of mRNA expression (correlation coefficient of dilutions versus densitometric values: $r = 0.966$). The mRNA level of each cytokine and iNOS is presented as relative units after

Table 2
Sequence of primers used in the RT-PCR

Cytokine	Forward primer	Reverse primer
IL-1β	5′ aat gaa ccg aga agt gg 3′	5′ ttc ttc gat ttg aga ag 3′
IL-10	5′ atg cat agc tca gca cta ctc tgt tgc ctg 3′	5′ tca ctt ttg cat ctt cgt tgt cat gta ggt t 3′
IL-12p40	5′ cac agc ttc tac cac gac ctc att 3′	5′ gct tgg agc aca ggg agt ata aca 3′
Il-12p35	5′ cac ctc agt ttg ggc agg agc ctc 3′	5′ ctc aga tag ctc atc att ctg tcg 3′
TNF-α	5′ cgg tag ccc acg ttg ta 3′	5′ tgg cct cag ccc act ct 3′
IFN-γ	5′ cct caa aga taa cca ggt c 3′	5′ cgc ttt ctg agg tta gat t 3′
iNOS	5′ tag agg aac atc tgg cca gg 3′	5′ tgg cag ggt ccc ctc tga tg 3′
GAPDH	5′ gag atg atg acc ctt ttg gc 3′	5′ gtg aag gtc gga gtc aac g 3′

GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

normalization to the observed GAPDH level. In addition, a change of 40% in relative units from the basal medium control expression was arbitrarily established as a requirement for the expression to be considered up or down regulation.

2.7. Nitric oxide production

$CD13^+$ or $CD172a^+$ cells were incubated with HK-BCG, BBO-MZ or medium for 96 h and the production of nitric oxide was determined. As a positive control for induction of nitric oxide production, the cells were also incubated in the presence of rBo-IFN-γ (50 U/ml) plus rHu-TNF-α (2500 U/ml). *S*-Methylisothiourea (SMT, 1 mM, Calbiochem, San Diego, CA, USA) was used as a competitive inhibitor of L-arginine to demonstrate the specificity of the RNI pathway (Granger et al., 1988). The concentration of nitrite, the stable oxidized form of nitric oxide, in the supernatant of the two populations was measured by the Griess reaction (Ding et al., 1988) as previously described (Goff et al., 1996). The results are shown as the mean micromolar concentration of nitrite in triplicate cultures.

3. Results

3.1. Identification, isolation and morphological characterization of myeloid cell populations from bovine spleen

The frequency of $CD13^+$ and $CD172a^+$ cells was determined in SMC and PBMC of calves. $CD13^+$ cells were absent from peripheral blood (Fig. 1A), but constituted approximately 6% of the total spleen cell population (Fig. 1B). The percentage of $CD172a^+$ cells was approximately 20% both in peripheral blood (Fig. 1C) and spleen (Fig. 1D). These results clearly demonstrate the differential distribution of $CD13^+$ cells between spleen and peripheral blood.

Total SMC were used for selection of either $CD13^+$ or $CD172a^+$ cells using mAb CC81 and CC149, respectively. The purity of the positively selected populations was assessed by FACS and ranged from 90 to 95% in both phenotypes (Fig. 1E and 1F). It is noteworthy that approximately 16% of the $CD13^+$ cells co-expressed CD172a and approximately 17% of the $CD172a^+$ cells co-expressed CD13 (Fig. 1G and H), demonstrating relatedness but, also, heterogeneity of the two populations.

It was of interest to determine whether these phenotypic differentiation molecules were influenced by culture conditions. Fig. 2 shows that both CD13 and CD172a surface markers were still expressed on the surface of the cells even after 96 h of culture.

Both populations were purified and morphology was assessed by staining the cells with Giemsa (Fig. 3). $CD13^+$ cells presented typical veiled DC morphology (Fig. 3A) whereas $CD172a^+$ cells showed typical monocyte morphology (Fig. 3B). In addition, both cell populations had similar capacity to bind antibody-sensitized sheep erythrocytes (data not shown) indicating the presence of Fc receptors.

3.2. Phenotypic characterization of $CD13^+$ and $CD172a^+$ populations

The expression of a variety of leukocyte differentiation-specific surface molecules on the surface of $CD13^+$ and $CD172a^+$ populations was determined by flow cytometry. Both populations expressed high levels of CD205 (WC6) and MHC class II (Figs. 4A and B, 5A and B). In addition, both $CD13^+$ and $CD172a^+$ populations were positive for CD11c (Fig. 4F and 5F) indicating a myeloid origin of the cells. Interestingly, the two populations were negative for CD11a (Fig. 4D and 5D) suggesting no expression of LFA-1 integrin on their surface. $CD13^+$ cells were negative for CD14 and the majority were negative for CD11b

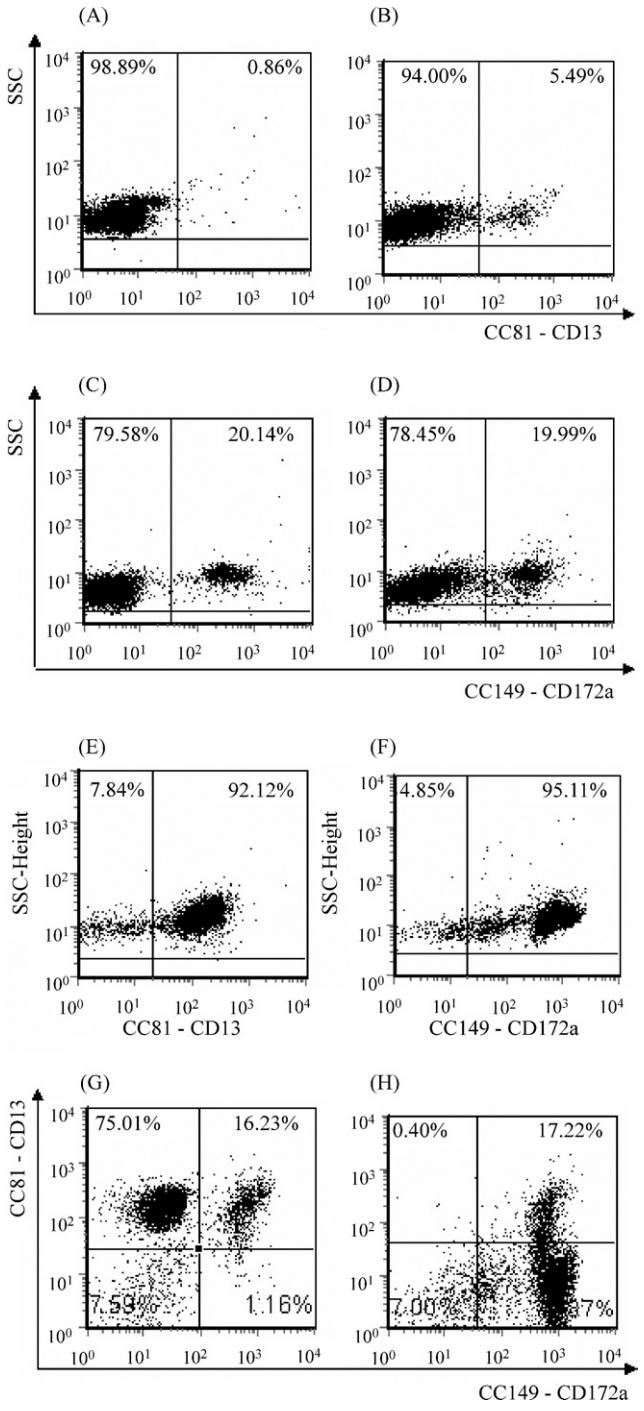

Fig. 1. Flow cytometry results representative of three calves. Panels A and B show the frequency of $CD13^+$ cells in peripheral blood and spleen, respectively. Panels C and D show the frequency of $CD172a^+$ cells in peripheral blood and spleen, respectively. Panels E and F show the purity of $CD13^+$ and $CD172a^+$ selected populations, respectively. Panel G demonstrates that approximately 16% of the $CD13^+$ selected cells are $CD172a^+$ whereas panel H shows that approximately 17% of the $CD172a^+$ selected cells are $CD13^+$, respectively.

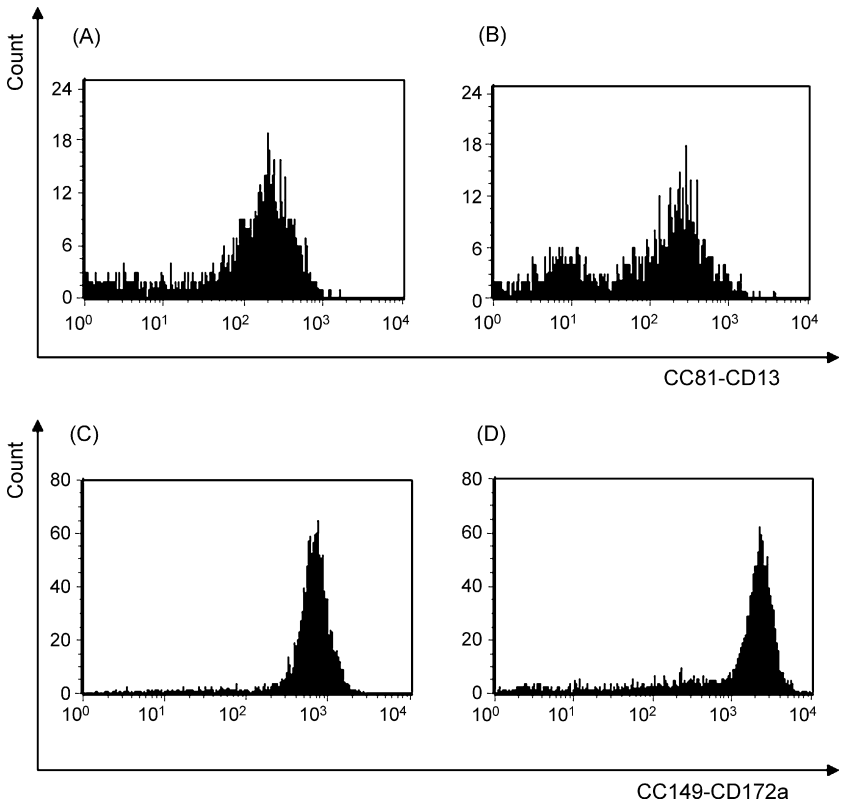

Fig. 2. Expression of CD13 and CD172a on the surface of selected splenic $CD13^+$ and $CD172a^+$ populations, respectively, under culture conditions. Panels A and B show the expression of CD13, using mAb CC81, on $CD13^+$ cells at time zero (just after selection) and 96 h of culture, respectively. Panels C and D show the expression of CD172a, using mAb CC149, on $CD172a^+$ cells at time zero (just after selection) and 96 h of culture, respectively.

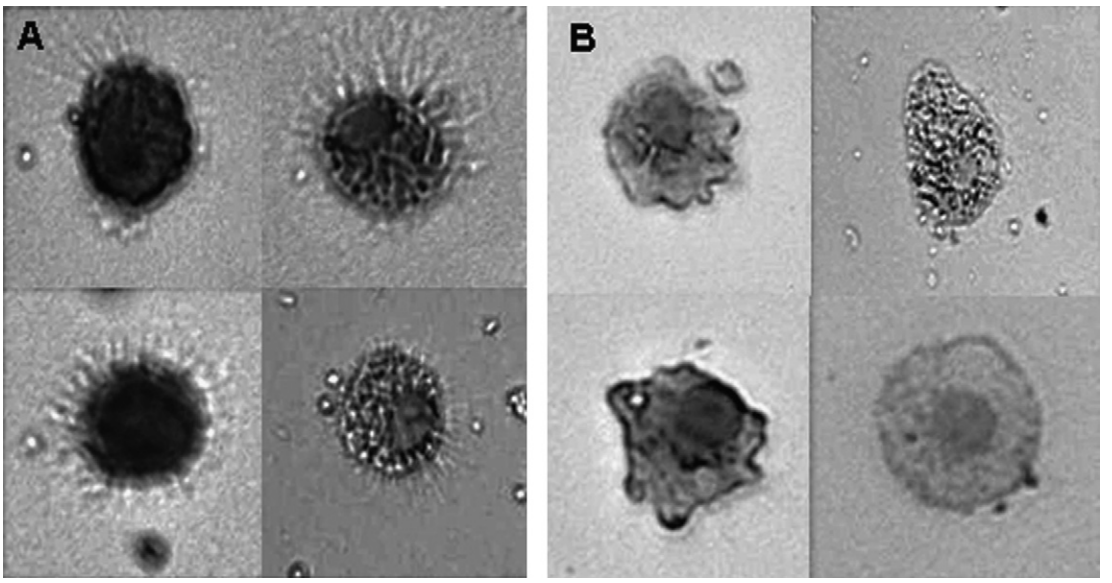

Fig. 3. $CD13^+$ and $CD172a^+$ populations were cultured in Iscove's medium for 96 h and cell morphology was assessed by Giemsa staining. Panels A and B show the morphology of $CD13^+$ cells and $CD172a^+$ cells, respectively (400×).

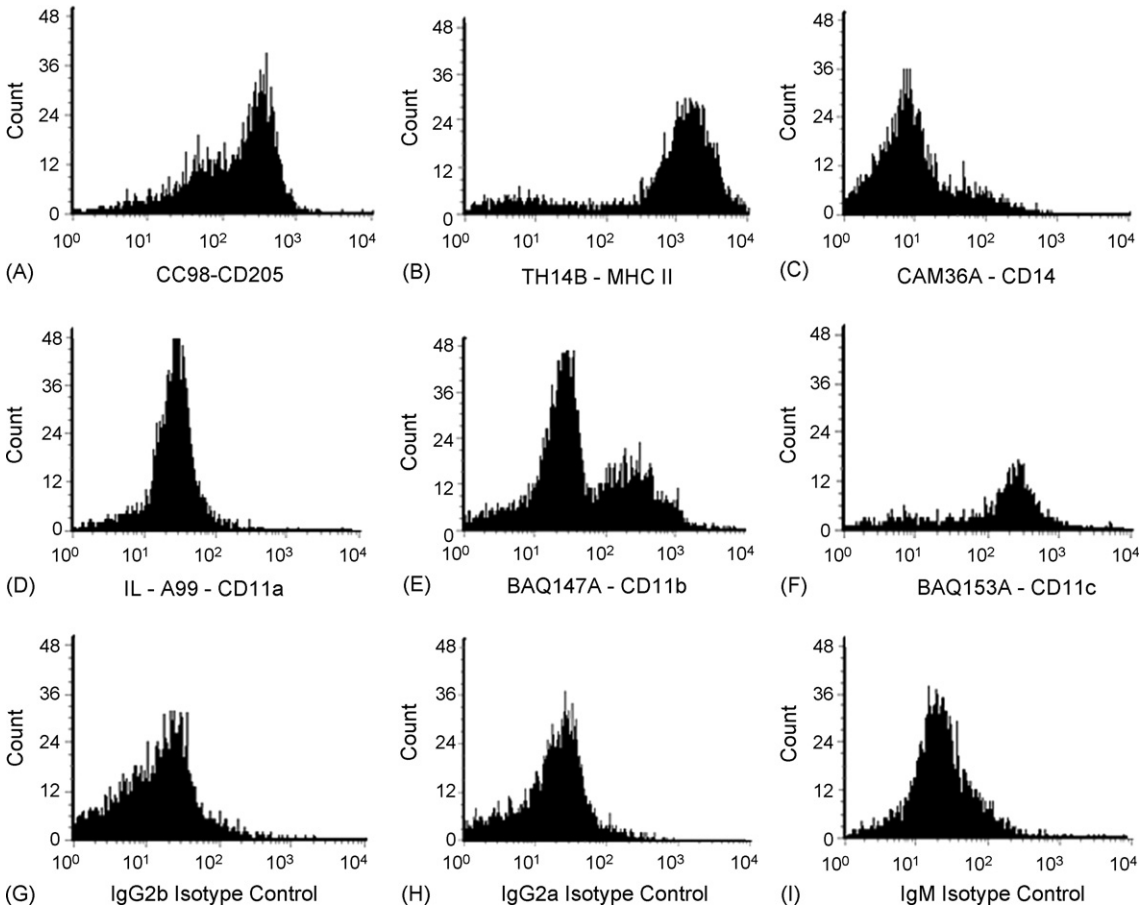

Fig. 4. $CD13^+$ selected cells were freshly stained with different mAbs for leukocyte differentiation-specific surface molecules and isotype-specific secondary antibodies. The staining intensity of the cells is shown in the histograms. Panels A to F show the staining for CD205, MHCII, CD14, CD11a, CD11b, and CD11c, respectively. Panels G, H and I show the isotype controls.

(Fig. 4C and E). In contrast, the majority of $CD172a^+$ cells were positive for both surface markers (Fig. 5C and E), confirming that the two subsets of myeloid cells are not phenotypically uniform cell populations. The minority subpopulation that appears as a shoulder in histogram (Fig. 4E) co-expresses CD11b and CD172a (data not shown).

3.3. RT-PCR for iNOS and nitric oxide production

$CD13^+$ and $CD172a^+$ populations were stimulated with either HK-BCG or BBO-MZ and the level of mRNA for iNOS was assessed by RT-PCR. $CD13^+$ cells produced only a modest level of iNOS message that was not modulated in the presence of the stimulants. In contrast, $CD172a^+$ cells produced abundant iNOS message that was up-regulated in the presence of mycobacterial and babesial stimulants (Fig. 6A). The relatively high amount of iNOS mRNA expressed by $CD172a^+$ cells even in the absence of stimulants demonstrated an intrinsic capacity of these cells to produce iNOS mRNA. Nitric oxide production paralleled the iNOS mRNA expression where $CD13^+$ cells did not produce any nitrite in the presence of stimulants whereas $CD172a^+$ cells produced 28.1 and 6.7 μM of nitrite in the presence of HK-BCG and BBO-MZ, respectively. Adding rBo-IFN-γ plus rhu-TNF-α to $CD13^+$ cells induced only a low level of nitrite (6.8 μM) whereas the same cytokines induced 58.8 μM of nitrite in $CD172a^+$ cells.

3.4. RT-PCR for cytokines

$CD13^+$ and $CD172a^+$ populations were incubated with HK-BCG and BBO-MZ, and the level of mRNA for IL-1β, IL-10, IL-12p40, IL-12p35, IFN-γ and TNF-α was determined at 4 and 8 h (Fig. 7). $CD172a^+$ cells expressed relatively strong IL-10 message in response to

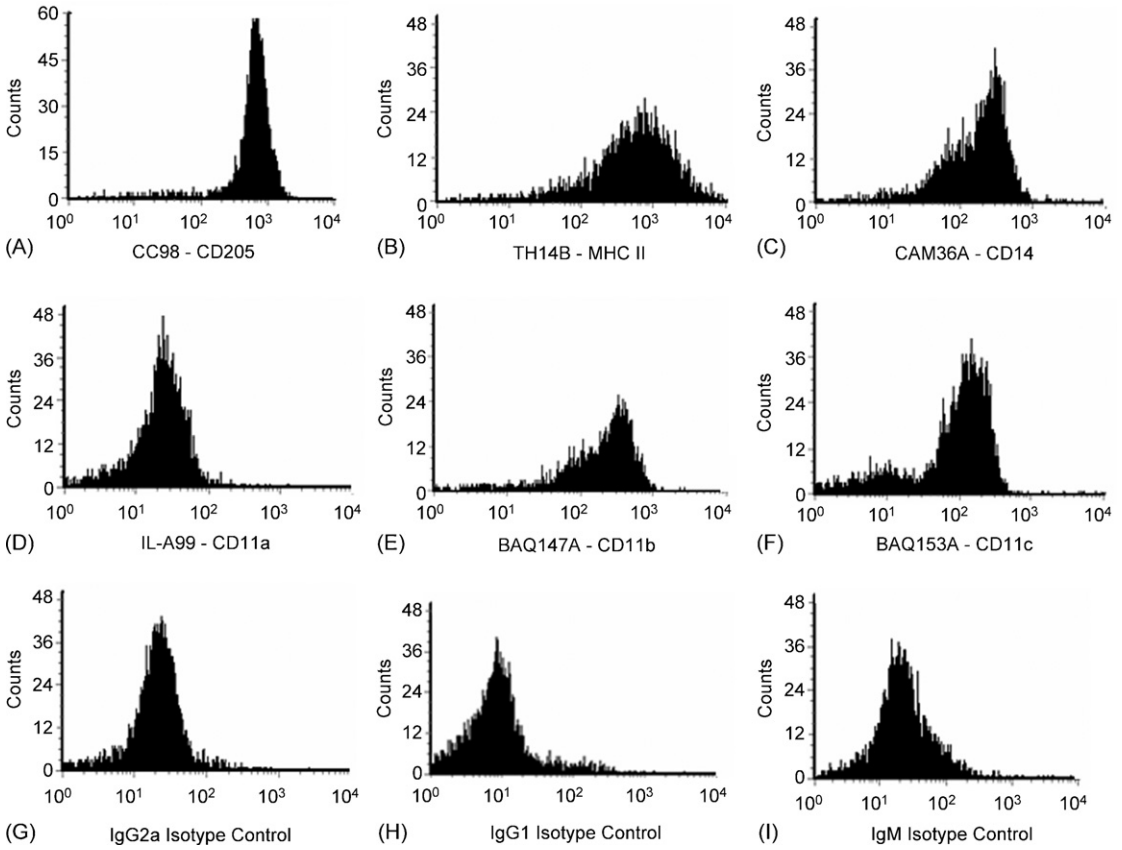

Fig. 5. CD172a$^+$ selected cells were freshly stained with different mAbs for leukocyte differentiation-specific surface molecules and isotype-specific secondary antibodies. The staining intensity of the cells is shown in the histograms. Panels A to F show the staining for CD205, MHCII, CD14, CD11a, CD11b, and CD11c, respectively. Panels G, H and I show the isotype controls.

microbial stimulants, particularly HK-BCG, in contrast to the CD13$^+$ cells where little or no IL-10 message was detected. CD13$^+$ cells expressed a modest amount of IL-12 p40 message in response to each stimulant at 8 h. However, at 4 h, IL-12 p40 transcripts were observed in CD172a$^+$ cells in response to microbial stimulation. This was the only difference in mRNA expression kinetics among the cytokines examined. Both cell populations expressed similar levels of IL-12p35 message, and it was not modulated by the presence of the stimulants. CD172a$^+$ cells expressed a higher level of IL-1β mRNA than CD13$^+$ cells, and it was up-regulated by microbial stimulation. CD172a$^+$ cells expressed strong levels of TNF-α message only in the presence of HK-BCG whereas CD13$^+$ cells expressed TNF-α mRNA in the presence of HK-BCG, BBO-MZ and medium. There was no detectable level of IFN-γ mRNA in either cell populations indicating that neither bovine splenic DC nor monocytes produce IFN-γ or that if there were any contaminating lymphocytes, they were not expressing IFN-γ message.

4. Discussion

In this paper we describe the isolation and partial characterization of two myeloid cell populations from bovine spleen, one with characteristics of a monocyte and another resembling a resident DC population. The two populations were purified using either CD172a or CD13 surface markers, respectively. CD172a is a member of the recently described family of signal regulatory proteins (SIRP) (van Beek et al., 2005) whereas CD13 is a cell-surface alanyl aminopeptidase (Riemann et al., 1999). Stable surface expression of both myeloid markers was demonstrated on the selected cells for at least 96 h of culture. Ligation of CD13 and CD172 by specific antibody used in the selection protocol did not affect the activation state or surface expression of other phenotypic molecules since the data presented here is similar to that reported by Zhuang et al. (2006) who used a depletion protocol to purify their cell populations. The purification procedure used in this study did not involve culture of the cells in the

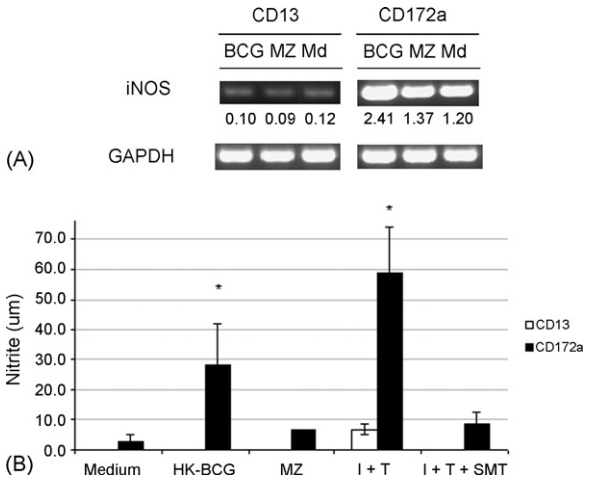

Fig. 6. Results of RT-PCR for iNOS mRNA and nitric oxide production by $CD13^+$ and $CD172a^+$ cell populations. Panel A shows the RT-PCR results of $CD13^+$ and $CD172a^+$ cells incubated for 8 h with either HK-BCG (50 μg/ml) or BBO-MZ (10:1) or medium (Md). GAPDH was used as the house keeping gene. The mRNA level is presented as relative units after normalization to the observed GAPDH level. Panel B shows the production of nitrite by $CD13^+$ (open bars) and $CD172a^+$ (black bars) cells incubated for 96 h with either medium, HK-BCG (50 μg/ml), BBO-MZ (10:1), rBo-IFN-γ (I) (50 U/ml) plus rHu-TNF-α (T) (2500 U/ml) or I plus T plus the nitric oxide inhibitor SMT (1 mM). The data represent the mean of three experiments. The Student's *t*-test was used to determine statistically significant differences in nitric oxide production $P < 0.05$ (*).

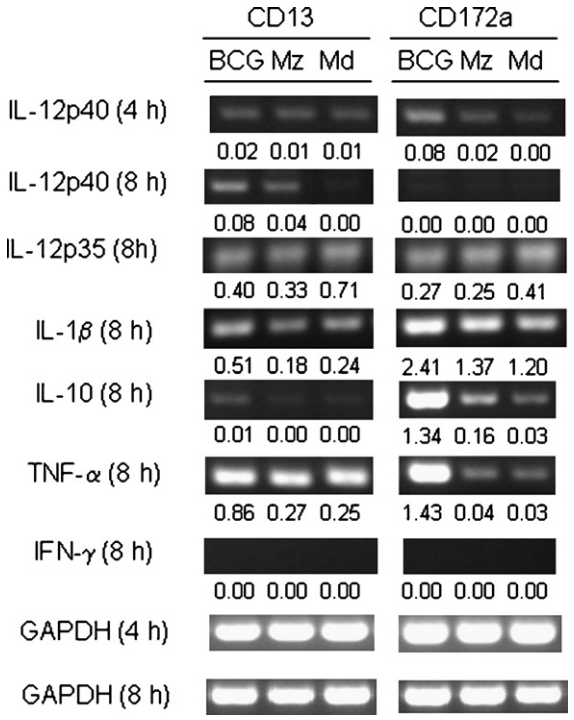

Fig. 7. RT-PCR results for cytokine mRNA expression by $CD13^+$ and $CD172a^+$ cell populations in the presence of either HK-BCG (50 μg/ml), BBO-MZ (10:1) or medium (Md). The mRNA level of each cytokine is presented as relative units after normalization to the observed GAPDH level.

presence of selected exogenous cytokines, thus the function of the cells was experimentally unbiased and reflective of their ex vivo activation state. After selection, the two populations appeared as two related but distinct phenotypes. Although related, they also differed in morphology, ability to produce nitric oxide and in the pattern of cytokine mRNA expression.

$CD13^+$ cells have been previously described in bovine afferent lymph (Howard et al., 1997) and here we demonstrate the presence of this phenotype in bovine spleen but its apparent absence in peripheral blood. CD13 and CD14 are markers for myeloid precursor cells with CD13 expression on DC being limited to immature cells (Bendriss-Vermare et al., 2001). Our data suggest that CD13 is retained while in secondary lymphoid organs, and thus may be a marker for at least one immature myeloid DC population in cattle. The data presented here is in agreement with the recent observation that CD13 expression on a bovine splenic DC population decreases as immature DC differentiate in the presence of IL-4, GM-CSF, CD40L and Flt3L (Zhuang et al., 2006).

Morphologically, $CD13^+$ cells displayed a veiled appearance confirming the intrinsic DC characteristic of this population whereas $CD172a^+$ cells presented typical monocyte morphology.

Phenotype characterization of $CD13^+$ and $CD172a^+$ selected cells revealed two distinct but related populations: $CD172a^+CD13^{+/-}CD14^+CD11a^-CD11b^{+/-}CD11c^+$ and $CD13^+CD172a^{+/-}CD14^-CD11a^-CD11b^{+/-}CD11c^+$. Phenotype plasticity of DC populations has been previously described by a number of investigators (Egner and Hart, 1995; Howard et al., 1997; Henri et al., 2001; Shortman and Liu, 2002). However, the expression of CD11b and CD14 on $CD172a^+$ cells provides further evidence that this is a monocyte/macrophage population (McKeever et al., 1991; Banchereau et al., 2000). CD11b expression on $CD13^+$ cells was restricted to the minority population that co-expressed CD172a reflecting the common lineage of these myeloid cells. CD11b in conjunction with CD18 forms Mac-1, a β2 integrin involved in cellular adhesion, migration and recognition (Iwasaki, 2003; Bimczok et al., 2005). Therefore, variable expression of CD11b may reflect a dynamic state where some DC are recent arrivals to the spleen, still expressing these adhesion molecules as well as CD172a, where others have become a resident population and have terminated expression of these markers while still

expressing CD13. $CD13^+$ and $CD172a^+$ populations were positive for CD11c, but interestingly $CD13^+$ cells were negative for CD11a contrasting the results previously described in afferent lymph (Howard et al., 1997). This may reflect a situation where the kinetics of CD11a down-regulation is quick and tightly regulated after being localized to the spleen.

Both cell populations were positive for CD205 (WC6) as previously described by (Gliddon et al., 2004) using afferent lymph veiled cells and peripheral blood cells. Moreover, $CD13^+$ and $CD172a^+$ cells were also positive for MHC class II, indicating that both cell populations have the ability to present antigen, as previously shown for migrating DC in afferent lymph (Howard et al., 1997; Gliddon et al., 2004). However, MHC II expression was greater in the $CD13^+$ population providing further evidence that this population is an immature DC population in the process of differentiation to a professional antigen-presenting cell. Although not included here, one might also expect to demonstrate increased expression of CD40 as well (reviewed by Hope et al., 2004).

The $CD172a^+$ cells expressed iNOS mRNA when placed in culture with increasing expression and nitric oxide production when in the presence of HK-BCG or BBO-MZ. In contrast, $CD13^+$ cells expressed only a modest amount of iNOS mRNA and they were unable to produce nitric oxide in the presence of mycobacterial and babesial stimulants. Given the fact that reactive nitrogen intermediates have microbicidal activity against a variety of microorganisms, including *M. bovis* BCG (Flynn and Chan, 2003) and *B. bovis* (Johnson et al., 1996; Shoda et al., 2000), the data suggest that $CD172a^+$ cells are better effector cells than $CD13^+$ cells consistent with previously published data comparing bovine blood monocyte-derived macrophages with monocyte-derived DC and their nitric oxide response to bacterial and CpG stimulation (Werling et al., 2004). The fact that $CD13^+$ cells expressed a low amount of mRNA for iNOS but produced no nitric oxide suggests the presence of a mechanism of post-transcriptional regulation of iNOS in this population (Kleinert et al., 2004).

The cytokine mRNA results show that there are qualitative and quantitative differences in IL-10, IL-12p40, IL-1β, and TNF-α mRNA expression between $CD13^+$ and $CD172a^+$ populations. The most striking difference between the two populations was in the expression of IL-10 and IL-12p40 mRNAs. IL-12 is one of the key cytokines required for priming a Th1 immune response whereas IL-10 is a cytokine related to a Th2 immune response (Mosmann et al., 1986; Mosmann and Coffman, 1989). Thus, it was of interest to determine the level of mRNA expression for these two cytokines in $CD13^+$ and $CD172a^+$ cells. The results indicated that IL-12 p40 transcripts were associated with both cell populations in the presence of either mycobacterial or babesial stimulation. However, the kinetics of expression was different with the peak of IL-12p40 mRNA expression at 4 h in $CD172^+$ cells whereas it was at 8 h in $CD13^+$ cells. IL-12p40 transcripts associated with a $CD13^+$ phenotype was previously observed in bovine lymph nodes (Stephens et al., 2003), and here we show that splenic $CD13^+$ cells express IL-12p40 message but are unable to express detectable IL-10 message. In contrast, $CD172^+$ cells expressed a high level of IL-10 mRNA. These results are also in agreement with previous data using $CD172^+$ adherent cells in culture on plastic (Goff et al., 1998b) and when comparing blood derived macrophages with DC (Werling et al., 2004). It is also important to note that the pattern of IL-10 and IL-12p40 mRNA expression by $CD13^+$ and $CD172a^+$ cells was consistent between mycobacterial and babesial stimulants.

It has been demonstrated that IL-1β induces DC to produce IL-12 (Wesa and Galy, 2001) and our results are in agreement where both IL-1β and IL-12 p40 expression were up-regulated in both cell populations in response to microbial stimulation. Although IL-12 p40 expression was similar in both populations, TNF-α mRNA expression was strongly up-regulated by HK-BCG in the $CD172^+$ population as previously described (Aldwell et al., 1997). Thus, the production of IL-1β, and TNF-α by $CD13^+$ and $CD172^+$ cells contribute to a type 1 response with a dichotomous role for $CD172a^+$ cells involving also the modulation of inflammatory cytokines by producing IL-10.

The IL-12 data did not provide enough evidence for determining whether the induction involved IL-12 p40 only or functional heterodimeric p70, although p35 message did not appear to be up-regulated. This study was done in the absence of T-cell interaction, which would likely affect the IL-12 results. DC interaction with T-cells via CD40 ligand has been shown to be required for functional p70 production (Cella et al., 1996; Stephens et al., 2003). Nevertheless, IL-12 p40 alone has been shown to be produced early after an infection and either by itself or as a dimer with subunits other than p35 i.e., p19 to form IL-23, to contribute to innate immunity involving IFN-γ production (reviewed by Abdi, 2002). Bovine NK cells were recently shown to produce IFN-γ in vitro in response to DC infected with *M. bovis* BCG (Hope et al., 2002) and we have also recently demonstrated that bovine splenic NK cells

expand during a *Babesia* infection (Goff et al., 2003a) and that naïve spleen NK cells produce IFN-γ in vitro after incubation with IL-12-containing supernatants from *B. bovis*-exposed monocytes (Goff et al., 2006). The assay for bovine IL-12 was however, limited to detecting p40. It is conceivable that microbial interaction with either splenic monocytes or DC induces p40 production that alone results in the induction of IFN-γ by NK cells (studies ongoing in our laboratory).

Based on the morphology, cell localization, phenotype, and cytokine profile with nitric oxide response to microbial stimulation, we conclude that $CD13^+$ cells are genuine immature splenic DC whereas $CD172^+$ cells are monocytes circulating through the spleen that can differentiate to a DC under appropriate conditions.

Acknowledgements

We appreciate the excellent technical support provided by Kim Kegerreis and Paul Lacy and for the care of animals provided by Ralph Horn and Duane Chandler. This study was supported financially by USDA-ARS-ADRU-CRIS no. 5348-32000-010.

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